Detection of novel divergent arenaviruses in boid snakes with inclusion body disease in The Netherlands

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Arenaviruses are bi-segmented negative-stranded RNA viruses, which were until recently only detected in rodents and humans. Now highly divergent arenaviruses have been identified in boid snakes with inclusion body disease (IBD). Here, we describe the identification of a new species and variants of the highly divergent arenaviruses, which were detected in tissues of captive boid snakes with IBD in The Netherlands by next-generation sequencing. Phylogenetic analysis of the complete sequence of the open reading frames of the four predicted proteins of one of the detected viruses revealed that this virus was most closely related to the recently identified Golden Gate virus, while considerable sequence differences were observed between the highly divergent arenaviruses detected in this study. These findings add to the recent identification of the highly divergent arenaviruses in boid snakes with IBD in the United States and indicate that these viruses also circulate among boid snakes in Europe.

Arenaviruses are negative single-stranded RNA viruses containing two segments: small (S) and large (L). The S segment is approximately 3.5 kb and encodes the viral nucleocapsid protein (NP) and the glycoprotein (GP), while the L segment is approximately 7.2 kb and encodes the RNA-dependent RNA polymerase (L) and a small ring-domain-containing protein (Z) (Buchmeier et al., 2007). Based on genetic differences and geographical distribution, arenaviruses are divided into two major lineages, Old and New World arenaviruses (Buchmeier et al., 2007; Charrel et al., 2008). At present, 24 species of the genus Arenavirus have been recognized by the International Committee on Taxonomy of Viruses (ICTV) (ICTV, 2011). In humans, infection with arenaviruses can cause severe disease including haemorrhagic fever and aseptic meningitis, but infections can also occur asymptotically (Moraz & Kunz, 2011). Rodents are considered as the main reservoir of arenaviruses and multiple outbreaks of haemorrhagic fever in humans have been associated with the presence of the virus in a rodent species (Charrel & de Lamballerie, 2010). In addition to the presence of arenaviruses in rodents and humans, highly divergent arenaviruses have been isolated recently from boa constrictors (Boa constrictor) and an annulated tree boa snake (Corallus annulatus) with inclusion body disease (IBD) (Stenglein et al., 2012).

IBD has been reported in multiple species, mainly in snakes of the family Boidae and Pythonidae (Chang & Jacobson, 2010; Vancraeynest et al., 2006). Clinical signs of this disease include abnormalities of the central nervous system and regurgitation, and most snakes die within weeks to months after the first observation of disease (Chang & Jacobson, 2010). Prior to the discovery of arenaviruses in boid snakes with histological evidence of IBD, diagnosis of the disease was based on the presence of cytoplasmic inclusions in haematoxylin and eosin stained tissue sections of affected snakes (Chang & Jacobson, 2010). Until recently, the aetiological agent(s) causing IBD had remained elusive, but the detection of arenaviruses in samples of snakes with histological evidence of the disease implicates these viruses as candidate aetiological agents of IBD (Stenglein et al., 2012).

In the present study, we performed random PCR in combination with next-generation sequencing on kidney samples of eight captive boid snakes that were diagnosed with IBD in The Netherlands. Tissues were collected from...
eight privately owned snakes (all from different owners), one emerald tree boa (*Corallus caninus*) and seven boa constrictors (*Boa constrictor*), of which two were albino variants, that were presented to the Department of Pathobiology of the Faculty of Veterinary Medicine, Utrecht University, The Netherlands for diagnostic necropsy and histopathology. All animals of this study were diagnosed to have IBD by histopathology based on eosinophilic intracytoplasmic inclusions that were found in various organs, including the kidneys, of all snakes. Tissues were collected aseptically and stored at −70 °C until further processing. Kidney samples were subsequently defrosted and homogenized using a FastPrep-24 (MP Biomedicals) in Hank’s balanced salt solution containing 0.5 % lactalbumin, 10 % glycerol, 200 U ml⁻¹ penicillin, 200 μg ml⁻¹ streptomycin, 100 U ml⁻¹ polymyxin B sulfate, 250 μg ml⁻¹ gentamicin, and 50 U ml⁻¹ nystatin (ICN Pharmaceuticals). Supernatant from homogenates was filtered and treated with nucleases (van den Brand et al., 2012; van Leeuwen et al., 2010). Subsequently, RNA and DNA were extracted using the Nucleospin RNA XS kit (Machery-Nagel) and the High Pure Viral Nucleic Acid kit (Roche) according to the instructions of the manufacturer. By random PCR in combination with next-generation sequencing using a 454 GS Junior instrument (Roche), random sequences of the samples were obtained as described previously (van den Brand et al., 2012). In brief, after first and second strand synthesis, random PCR amplification was performed and PCR products were purified with the MiniElute PCR purification kit (Qiagen). Unique sequence tags were added to PCR products of each sample using the GS FLX Titanium Rapid Library MID Adaptors kit. Subsequently, a library of DNA fragments was prepared using a GS FLX Titanium library preparation kit (454 Life science, Roche), and this library of DNA fragments was sequenced on a 454 GS Junior instrument (454 Life science, Roche). Adaptor and primer sequences were removed from all reads, and obtained reads were assembled using *de novo* assembly in CLC Genomics Workbench 5.5.1 (CLC Bio), analysed according to nucleotide (contigs and singletons) and translated by nucleotide BLAST. Sequences were classified into viruses, phages, bacteria and eukaryotes based on the taxonomic origin of the best-hit sequence using MEGAN software (Huson et al., 2011). E-values of 10⁻¹⁰ were used as the cutoff value of significant virus hits for BLASTN and BLASTX, respectively. Between 5000 and 18 000 individual sequences, mostly of eukaryotic origin, were amplified in each tissue sample. In all samples, arenavirus-like sequences were detected. In addition, in four out of eight snakes, including the emerald tree boa, sequences with 40–82 % identity (sequence length 255–619 nt) at the amino acid level to a *Python molurus* endogenous retrovirus were detected. In one of the three boa constrictors, sequences were detected that showed 94–99 % identity (sequence length 436 nt) at the nucleotide level to snake adenovirus 1 strain 145/88 (Table 1).

In addition to next-generation sequencing, a near complete arenavirus genome sequence was determined from boa constrictor 3 (Boa Av NL; GenBank accession numbers KC508669 and KC508670). To this end, specific primers designed on the obtained 454-sequencing reads were used to obtain partially overlapping PCR amplicons of 400-1000 nt of the arenavirus genome, using AmpliTaq Gold DNA polymerase (Roche), according to instructions of the manufacturer. Amplicons were ligated into the pCR 2.1 Topo vector (Invitrogen) and sequences of at least two clones in two directions were obtained as described previously (van Leeuwen et al., 2010). Primer sequences are available upon request. The near complete divergent arenavirus genome was tentatively named Boa Av NL B3 (GenBank accessions KC508669 and KC508670). In both the S (3351 nt) and L (6933 nt) segment of the Boa Av NL B3 virus, two opposite-sense open reading frames (ORF) were identified arranged similar to the recently described arenaviruses of boid snakes (Stenglein et al., 2012). The ORF at the 5' end of the S segment, encoding the GP, consists of 1284 nt and the ORF at the 3' end, encoding the NP, consists of 1755 nt with an inter-ORF sequence of 150 nt. The small (Z) and large (L) ORF on the L segment consist of 351 nt and 6198 nt respectively with an inter-ORF sequence of 154 nt.

Nucleotide sequences of the coding regions of the NP and L genes of Boa Av NL 3 virus were aligned with the divergent arenaviruses recently isolated from boid snakes, Table 1. Viral sequences identified in tissue samples of boid snakes

<table>
<thead>
<tr>
<th>Study no. of snake</th>
<th>Snake species</th>
<th>Total no. of sequence reads</th>
<th>Viruses detected by next-generation sequencing (no. of single sequences)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Emerald tree boa</td>
<td>18035</td>
<td>Arenavirus (3), Endogenous retrovirus (2)</td>
</tr>
<tr>
<td>2</td>
<td>Boa constrictor</td>
<td>11578</td>
<td>Arenavirus (561), Endogenous retrovirus (26)</td>
</tr>
<tr>
<td>3</td>
<td>Boa constrictor</td>
<td>18315</td>
<td>Arenavirus (2364)</td>
</tr>
<tr>
<td>4</td>
<td>Boa constrictor (albino)</td>
<td>8678</td>
<td>Arenavirus (587), Endogenous retrovirus (16), Snake adenovirus (25)</td>
</tr>
<tr>
<td>5</td>
<td>Boa constrictor</td>
<td>7358</td>
<td>Arenavirus (13)</td>
</tr>
<tr>
<td>6</td>
<td>Boa constrictor</td>
<td>10011</td>
<td>Arenavirus (396), Endogenous retrovirus (21)</td>
</tr>
<tr>
<td>7</td>
<td>Boa constrictor</td>
<td>5153</td>
<td>Arenavirus (380)</td>
</tr>
<tr>
<td>8</td>
<td>Boa constrictor (albino)</td>
<td>9433</td>
<td>Arenavirus (256)</td>
</tr>
</tbody>
</table>
the California Academy of Science virus (CASV) and the Golden Gate virus (GGV) and with various other viruses of the genus *Arenavirus*. Multiple alignments were created using the CLUSTAL W method and phylogenetic analyses were performed using neighbour-joining with the p-distance model, 1000 bootstrap replicates, and otherwise default parameters in MEGA5 (Tamura *et al.*, 2011).

Furthermore, pairwise sequence identities between GGV, CASV and Boa Av NL B3 were calculated with MEGA5 software using default parameters.

Phylogenetic analyses of the nucleotide sequences of the NP and L ORFs of Boa Av NL B3 virus revealed that the sequences were most closely related to the GGV (Fig. 1a, b).
with pairwise nucleotide sequence identities between 66.4% and 77.4% for all the ORFs and pairwise deduced amino acid identities between 66.3% and 80.9% (Table 2). The virus was more distantly related to the CAS virus with pairwise nucleotide sequence identities between 42.3% and 60.0% for all the ORFs and pairwise amino acid identities between 51.1% and 58.4%. To further analyse the identity between the Boa Av NL B3 virus with the GGV and the CASV, a similarity plot of the nucleotide sequence of the ORFs and the inter-ORF region of both gene segments of these viruses was prepared using SimPlot 3.5.1 (Lole et al., 1999). These plots confirm the findings of the phylogenetic analysis and show that within ORFs regions with different similarity exist (Fig. 1d, e).

In conclusion, we have identified various variants of the highly divergent snake arenaviruses in all investigated samples of boid snakes with IBD. These findings confirm the results of Stenglein and colleagues who identified similar viruses in snakes with IBD and indicate that these viruses also circulate among boid snakes in Europe (Stenglein et al., 2012). However, Koch’s postulates need to be fulfilled for this virus using a cloned or purified virus to prevent potential contaminating agents as much as possible before it is clear whether this virus is the cause of IBD (Schumacher J et al., 1994; Wozniak et al., 2000). In addition, the identification of these highly divergent arenaviruses raises the question whether rodents are also carriers of these arenaviruses, as has been demonstrated for Old and New World arenaviruses (Charrel & de Lamballerie, 2010). The results of this study can be used for the development of diagnostic assays and suggest that design of intervention strategies, such as vaccination or the use of antivirals against the multiple species of IBD-associated arenavirus, should be focussed on development of either multivalent vaccines or on the development of vaccines that focus on the development of immunity against more conserved regions (Botten et al., 2010; Lee et al., 2011).

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### References


